

Diabetes-Induced Vascular Hypertrophy Is Accompanied by Activation of $\text{Na}^+\text{-H}^+$ Exchange and Prevented by $\text{Na}^+\text{-H}^+$ Exchange Inhibition

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Abstract—Vascular disease often involves vessel hypertrophy with underlying cellular hypertrophy or hyperplasia. Experimental diabetes stimulates hypertrophy of the rat mesenteric vasculature, and we investigated the hypothesis that this hypertrophy is associated with activation of $\text{Na}^+\text{-H}^+$ exchange (NHE) activity. We measured the NHE activity in isolated, intact blood vessels from control and streptozotocin-induced diabetic adult rats using concurrent myography and fluorescence spectroscopy. The role of inhibiting NHE activity in preventing the development of the mesenteric hypertrophy in streptozotocin-diabetic rats was investigated by administration of cariporide (100 mg/kg body weight per day in 3 doses by gavage) after induction of diabetes and subsequently determining vessel weight and structure. The weight of the mesenteric vasculature was not increased 1 week after streptozotocin treatment but was significantly increased by an average of 56% at 3 weeks. NHE activity in mesenteric arteries showed an enhanced maximal velocity (V_{max}) in diabetic vessels at 1 and 3 weeks (0.246 ± 0.006 and 0.238 ± 0.007 versus 0.198 ± 0.007 pH U/min) with no change in the apparent K_m . Moreover, NHE-1 mRNA in mesenteric arterioles at 3 weeks after streptozotocin treatment was increased by >60% (55.8 ± 6.4 versus 91.3 ± 12.3 fg). Administration of cariporide significantly reduced mesenteric vascular weight, the wall/lumen ratio, and mesenteric extracellular matrix accumulation in the diabetic animals. Our study shows that diabetes in vivo correlates with elevated NHE activity and mRNA in the mesenteric vasculature and furthermore that inhibition of this system prevents the hypertrophic response. These data suggest that NHE may be a target for therapeutic modulation of vascular changes in diabetes. (*Circ Res.* 2000;87:1133-1140.)

Key Words: experimental diabetes ■ $\text{Na}^+\text{-H}^+$ exchange ■ vascular smooth muscle ■ vascular hypertrophy ■ cariporide

There are a number of clinical disorders that display a component of accentuated vascular smooth muscle growth, including essential hypertension,¹ atherosclerosis,² restenosis after coronary angioplasty,³ and diabetes.⁴ Significantly, enhanced $\text{Na}^+\text{-H}^+$ exchange (NHE) activity of erythrocytes, leukocytes, platelets, and fibroblasts has been found in both essential hypertension and type 1 diabetes.⁵ NHE activity is elevated in both cultured vascular smooth muscle cells (VSMCs)⁶ and vessels⁷ from genetically hypertensive rats. Growth factors⁸ and hormones, including vasoconstrictors,⁹ activate the NHE in numerous cell types, but the role of this transporter in cell growth remains controversial. NHE antagonists from the amiloride series block the proliferation of VSMCs with a potency identical to their activity against the antiporter.¹⁰ Furthermore, NHE inhibitors, including ethylisopropylamiloride and HOE-694, block the in vivo proliferation of VSMCs observed with the development of a

neointima after balloon angioplasty in the rat carotid artery.^{11,12} Further clarification of the role of the NHE transporter in the pathogenesis of these vascular proliferative diseases is therefore of importance, particularly in the in vivo setting.

Diabetes is associated with both microvascular and macrovascular disease, manifested as altered vascular morphology. VSMCs exposed to elevated glucose¹³ and aortic smooth muscle cells originating from diabetic rabbits or rats^{14,15} exhibit increased in vitro growth rates. Previous studies on cultured VSMCs exposed to high glucose demonstrated an increase in the rate of NHE.¹⁶ In the light of these results, it is possible that alterations in the NHE system might play a role in the development and/or maintenance of vascular disease in diabetes. However, findings in cultured cells may not necessarily reflect the in vivo situation. Thus, the present study investigated in detail the characteristics of NHE in

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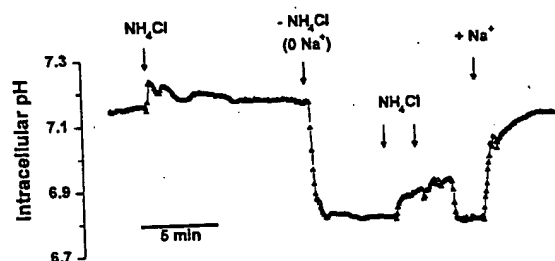


Figure 1. A typical trace showing measurement of pH_i in vascular smooth muscle of rat small arteries. Measurements were conducted in vessels mounted in a thermostatically controlled myograph and loaded with BCECF-AM in a bicarbonate-free PSS as described in Materials and Methods. Acidosis necessary to activate the NHE was induced by exposure to and removal of NH_4Cl , and the rate of recovery of pH_i after the reintroduction of sodium to the acidotic tissue was monitored. Buffer capacity was measured by pulsing low concentrations (1 and 2 mmol/L) of NH_4Cl as indicated by arrows. Actual pH_i values were determined by the nigericin-clamp technique at the end of each experiment.

intact mesenteric arteries from streptozotocin-induced diabetic rats. Specifically, the study tests the hypothesis that hyperglycemia and its *in vivo* metabolic consequences activate NHE in a vascular bed that we have previously shown to exhibit hypertrophy.¹⁷ We have also used the NHE inhibitor cariporide¹⁸ to determine whether inhibiting the vascular NHE can prevent or reduce the extent of the vascular hypertrophy occurring in this model of diabetes.

Materials and Methods

Experimental Solutions

Experiments were conducted in a physiological salts solution (PSS) that was bicarbonate free. Sodium replacement was with equimolar tetramethylammonium chloride. pH_i calibrations were by nigericin clamp with PSS containing nigericin (5 $\mu g/mL$) and 140 mmol/L KCl.

Generation of Diabetic Rats

Male Sprague-Dawley rats (Biological Research Unit, Baker Medical Research Institute, Prahran, Victoria, Australia) were used in this study. Ten-week-old rats weighing 250 to 300 g were randomized into control (nondiabetic) or diabetic groups. Rats were made diabetic by a single injection of streptozotocin (45 mg/kg body weight IV).

Mesenteric Vessel Isolation, Analysis, Mounting, and Tension Normalization

Mesenteric arterioles were processed and weighed as previously described.¹⁷ For studies of NHE by measurement of pH_i , 6 to 9 animals were used in each group, with 1 or 2 vessel segments from each rat. All vessels were passed through an identical work program, mounted in the myograph, and then studied at equivalent tensions. Vessels were set to an internal circumference at which they were held under tension. The resting tension-internal circumference relation was then determined using a computer program (G.A. McPherson, NORMALIZE, 1985 version).

Determination of NHE Activity

To evaluate NHE activity in mesenteric arterioles, the pH_i rate of recovery from an induced acidification, cellular buffer capacity of the mesenteric arterioles, and the calibration of the fluorescence to pH_i values were determined.

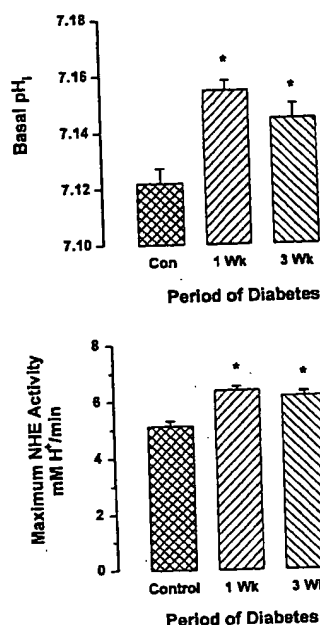


Figure 2. Effect of streptozotocin-induced diabetes on the basal pH_i and maximal rates of NHE activity in rat mesenteric arteries. Mesenteric arteries were obtained from adult rats 1 and 3 weeks after administration of vehicle or streptozotocin. Arteries were mounted in a myograph and loaded with BCECF-AM in a bicarbonate-free PSS. Vessels were taken through a contraction protocol, after which basal pH_i was determined. Experiments concluded with calibration of fluorescence and pH_i by the nigericin-clamp technique. Top, Basal pH_i values. Bottom, Maximal NHE activities determined from maximum rate of pH_i recovery after acid loading and mean buffering capacity (25.8 mmol/L H^+). Each result derives from 7 or more determinations in independent vessels. * $P < 0.05$ vs control.

Measurement of pH_i

pH_i was determined by monitoring the fluorescence of the pH -sensitive dye BCECF. For these measurements, the vessel (mounted in myograph) was incubated for 0.5 hours in BCECF-AM (10 $\mu mol/L$) in HEPES PSS.

Determination of Recovery Rate After Vessel Acidification and Buffer Capacity

Intracellular acidification was achieved using the NH_4Cl "prepulse" technique. The Na^+ -dependent recovery in the absence of bicarbonate has previously been characterized in vascular smooth muscle to be due solely to NHE.¹⁹ Actual NHE activity is the true proton extrusion rate and was calculated as the product of the rates of change in pH_i and tissue buffer capacity (β_i). Therefore, the intrinsic buffering capacity (β_i) of the tissues was measured using the method of Roos and Boron²⁰ as shown in Figure 1 and calculated using the equation $\beta_i = \Delta(NH_4^+)/\Delta pH_i$.

Data Analysis

Recorded fluorescence ratio values were converted into pH_i values using the calibration curve obtained for each experiment. The rate of change of pH_i after an acid load ($\Delta pH_i/second$) was calculated by using AcqKnowledge software (Waveform Data Analysis for Windows), version 2.02, by Biopac Systems, Inc. Using this program, rates of change along the recovery curve could be obtained at specified levels of pH_i . The transport rates at each level of pH_i were analyzed using a Hill plot in which $\log(V/V_{max} - V)$ is plotted against $[H^+]$. V_{max} for this calculation was defined operationally as the maximal transport rate observed after the readmission of Na^+ to

TABLE 1. Data Showing Effects of Streptozotocin-Induced Diabetes on pH_i and Properties of the NHE in Mesenteric Arteries

	Mesenteric Artery		
	Control	1-Week Diabetic	3-Week Diabetic
Maximum acidification (pH _i)	6.80±0.01	6.79±0.01 (NS)	6.79±0.02 (NS)
K _m ⁺ (pH _i)	6.97	6.97	6.97
V _{max} pH units/min	0.198±0.007	0.246±0.006†	0.238±0.007†
Intrinsic buffer capacity (β _i), mmol/L H ⁺	24.8±2.3	26.8±2.5 (NS)	26.7±2.0 (NS)
Hill coefficient	4.31±0.26	4.01±0.25 (NS)	5.01±0.47 (NS)

Logistic sigmoid function (minimum+[maximum-minimum]/1+exp[-k(x-x50)]).

†P<0.05.

acid-loaded tissues. This was the rate observed for the pH interval of ≈6.84 to 6.86. For this analysis, data from 6 to 9 experiments were pooled and 108 to 162 rate constants were calculated for each experimental group and used to generate the composite Hill plots. The resultant Hill coefficients (the slopes of these graphs) were compared for control and diabetic animals. We used our estimates of β_i at pH 6.8 to convert the observed rate of change in pH_i to an estimate of the actual H⁺ efflux rate (ΔH⁺ concentration/min).

All values are reported as mean±SE. Data between groups were analyzed using unpaired t test or the least significant difference method as specified. P<0.05 was deemed to be statistically significant.

Competitive Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Estimation of NHE-1 Gene Expression

Quantitative RT-PCR was used to determine an accurate level of mRNA in the pool of total RNA extracted from mesenteric arterioles.

The NHE-1 primers used were antisense 5'-TGT GTC TGT TAT AGG ACC GCA GCC-3' and sense 5'-CCA GCT CAT TGC CTT CTA CC-3' (2486 to 2730 [244 bp]). To determine the concentration of mRNA, increasing amounts of a specific NHE-1 competitor (as above but minus 2644 to 2676 [212 bp]) were added to the reaction (5 to 1000 fg), and the concentration of NHE-1 mRNA is equivalent to that concentration of competitor required to give the same intensity of radioactivity incorporation.

Administration of Cariporide and Quantification of Mesenteric Histomorphology

Sprague-Dawley rats weighing 200 to 250 g were randomized to receive either streptozotocin (45 mg/kg) or citrate buffer alone. All rats were given free access to standard rat chow containing 20% protein (Clark, King and Co). The study protocol used 4 groups of rats, as follows: controls, controls plus cariporide, diabetes, and diabetes plus cariporide. Rats were administered cariporide (100 mg/kg per day in 3 divided doses) by gavage; and control animals received water by the same route of administration. The study was approved by the Ethics Committee of the Austin and Repatriation Medical Center. For assessment of mesenteric structure, the vessels were removed, fixed in 10% formalin, and embedded in paraffin using standard procedures, and the medial layer of vessels and corresponding lumen areas were determined as previously described.²¹ Quantification of the area of the extracellular matrix (ECM) within the vessel wall was performed on trichrome-stained sections (Masson) using a computer-assisted image analysis system²² and was related as a ratio of a proportional area to the total area of media or adventitia.^{21,22}

Chemicals

BCECF-AM was purchased from Molecular Probes. HEPES and nigericin were purchased from Sigma Chemical Co. Cariporide (4-isopropyl-3-methylsulfonyl-benzoylguanidine methane sulfonate, HOE 642) was kindly provided as a gift by Aventis.

An expanded Materials and Methods section can be found in an online data supplement available at <http://www.circresaha.org>.

Results

Vascular Hypertrophy After Experimental Diabetes

The weight of the entire mesenteric vasculature of control and streptozotocin-treated rats was determined to confirm our earlier finding illustrating the occurrence and temporal aspects of the development of vasculature hypertrophy in this model.¹⁷ Control animals showed a small increase in vessel weight over the 2-week period, consistent with growth of the animals. Compared with their time-matched controls, there was no increase in mesenteric weight 1 week after streptozotocin treatment, but 3 weeks after treatment there was a 56.0% (P<0.001) increase in mesenteric weight, with final average weights being 35.7±1.2 mg (n=6) and 55.7±1.6 mg (n=7) in the control and streptozotocin-treated groups, respectively.

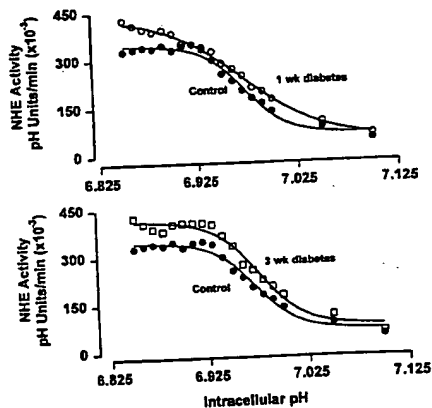


Figure 3. Effect of streptozotocin-induced diabetes on pH_i recovery rates in rat mesenteric arteries. Vessels were obtained from control animals (solid symbols) and those with streptozotocin-induced diabetes (open symbols) for 1 (top) and 3 (bottom) weeks. Vessels were mounted in a myograph and loaded with BCECF-AM for determination of pH_i. Rates of recovery were determined from instantaneous estimations as described in Materials and Methods. Data show rates of recovery as pH units per minute over the range of pH values.

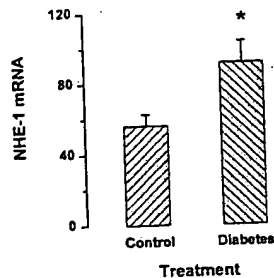


Figure 4. Determination of NHE-1 mRNA in mesenteric arteries. Total RNA was isolated from the mesenteric arteries of control or 3-week streptozotocin treated rats and NHE-1 mRNA determined by RT-PCR as described in Materials and Methods. Top, RT-PCR for NHE-1 from a control animal with increasing concentrations of competitor (0, 10, 50, 70, 100, 500, 700, 1000, and 5000fg). In the photograph, the upper (larger molecular weight) band is the NHE-1 and the lower the competitor. Bottom, Pooled results for analysis of vessels from 5 control and 4 diabetic animals. * $P < 0.05$.

Effect of Experimental Diabetes on NHE Activity

The parameters necessary to fully characterize the properties of NHE activity in a preparation are resting or basal pH_i , maximal rate of recovery from a maximally induced acidosis, and sensitivity of the transporter to intracellular protons. We systematically assessed each of the properties in the rat mesenteric arteries.

Measurements of Basal pH_i in Mesenteric Arteries

In mounted vessels prepared by exposure to a work protocol that normalized the preparations, we evaluated resting pH_i and NHE activity in mesenteric arteries at 1 and 3 weeks after the induction of diabetes. The resting pH_i in control mesenteric arteries measured in bicarbonate-free HEPES-PSS solution averaged 7.12 ± 0.005 . The resting pH_i of vessels from rats with diabetes established for both 1 and 3 weeks was

significantly increased to 7.15 ± 0.003 and 7.14 ± 0.006 , respectively ($P < 0.05$) (Figure 2).

Measurements of Maximal NHE Activity in Mesenteric Arteries

NHE activity was assessed from the rate of recovery of pH_i after the reintroduction of extracellular sodium to a vessel acidified by the ammonium chloride withdrawal technique conducted in sodium-free solution. An example of a typical experimental trace is shown in Figure 1. There was no significant difference ($P > 0.05$) in the level of acidification on NH_4Cl washout, which averaged 6.80 ± 0.004 across all experimental groups (Table 1). The maximal rate of recovery (V_{max}) averaged 0.198 ± 0.007 pH units/min in control mesenteric arteries (Table 1). The maximal rates of recovery were significantly increased ($P < 0.05$) after 1 and 3 weeks of diabetes, averaging 0.246 ± 0.006 and 0.238 ± 0.007 pH units/min, respectively (Table 1).

The intrinsic buffer capacity (β_i) at pH 6.8 in mesenteric arteries from control and diabetic rats averaged 24.83 ± 2.34 mmol/L H^+ in controls and 26.75 ± 2.50 mmol/L H^+ and 26.67 ± 1.95 mmol/L H^+ in 1-week and 3-week diabetic vessels, respectively (Table 1). As there was no significant difference among these estimates of β_i , data from control and diabetic mesenteric arteries were combined, and the overall mean value for β_i (25.8 mmol/L H^+) was used to calculate the effective H^+ efflux rates (H^+ efflux = $\Delta pH_i / \text{min} \times \beta_i$), which is the best estimate of NHE activity. The resultant estimates for H^+ efflux rates were 5.11 ± 0.18 mmol/L H^+ per minute in controls and 6.35 ± 0.16 and 6.14 ± 0.18 mmol/L H^+ per minute in 1-week and 3-week diabetic vessels, respectively (Figure 3). Proton efflux rates of both 1-week and 3-week diabetic vessels were significantly higher than those of control vessels.

Dependence of Na^+-H^+ Activity on pH_i in Mesenteric Arteries

To estimate the affinity of the NHE for intracellular H^+ , NHE activity (measured as the rate of recovery of pH_i after acid loading) was calculated at specific levels of pH_i . The relationship between NHE activity and pH_i was determined by obtaining the "instantaneous" slope of the recovery line at pH_i intervals of 0.05 pH units, from pH_i 6.85 to 7.0, and for 7.05 and 7.10 (18 points in all, for each experiment). The rate of change of pH_i was then

TABLE 2. Metabolic and Physiological Characteristics of Control and Diabetic Rats Administered Cariporide

Treatment	n	Body Weight, g	Mesenteric Weight, g	Heart Weight, g	Blood Glucose, mmol/L	Blood Pressure, mm Hg
Control	8	340 \pm 7	0.149 \pm 0.008	1.48 \pm 0.05	6.5 \pm 0.2	121 \pm 13
Control+cariporide	8	320 \pm 5	0.133 \pm 0.005	1.52 \pm 0.13	7.0 \pm 0.3	130 \pm 13
Diabetic	7	227 \pm 9*	0.235 \pm 0.019*	1.10 \pm 0.08*	21.5 \pm 0.8*	131 \pm 7
Diabetic+cariporide	10	227 \pm 8*	0.177 \pm 0.008†	1.22 \pm 0.04*	23.5 \pm 1.0*	123 \pm 3

Adults rats were rendered hyperglycemic by administration of streptozotocin, and control rats received vehicle. Subsets of rats in each group were administered cariporide (100 mg/kg per day) by gavage in 3 equal doses per day. Animals were euthanized at 3 weeks and the parameters shown below determined. Data are mean \pm SEM.

* $P < 0.01$ vs control.

† $P < 0.05$ vs diabetic.

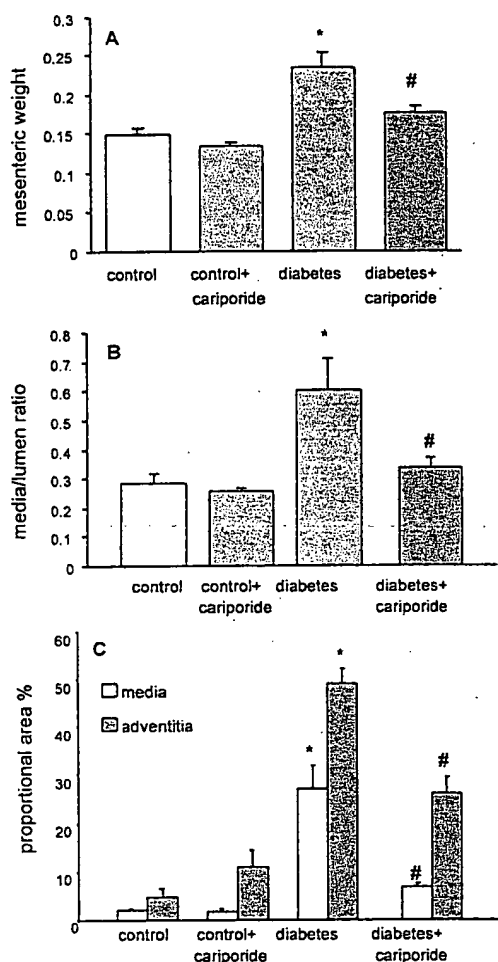


Figure 5. Inhibition of Na⁺/H⁺ exchange with cariporide prevents the development of mesenteric vascular hypertrophy in diabetic rats. Diabetes was induced by treatment with streptozotocin. Animals were given cariporide by gastric gavage 3 times daily for 3 weeks, and the mesenteric weights (top), media:lumen ratios (middle), and proportional area of matrix (defined as ratio of positively staining area to total area of media or adventitia [bottom]) were determined. There were 6 to 10 rats in each group. Values that are significantly different ($P < 0.01$) are indicated with * (control vs diabetes) and # (diabetes vs diabetes and cariporide).

plotted as a function of pH_i (Figure 5) and fitted to a logistic sigmoidal function $\{ \text{minimum} + [\text{maximum} - \text{minimum}] / 1 + \exp[-k(x - x_{50})] \}$. The dependence of NHE activity on intracellular H⁺ was steep and sigmoidal. There was no significant difference in the K_m values for mesenteric arteries from control or diabetic animals (Table 1), nor was there any significant change in the proton modifier site as indicated by the Hill coefficient (Table 1).

Determination of the Biochemical Mechanism of NHE Activation

Studies using cultured VSMCs demonstrated that the activation of NHE by treatment with high-glucose media for 24 hours was associated with increased mRNA for NHE-1.¹⁶

Because the levels of NHE-1 gene expression are generally quite low, we developed a competitive RT-PCR assay to determine the levels in the rat mesenteric arterioles. We determined NHE-1 mRNA in vessels from control and diabetic animals. NHE-1 mRNA per microgram total mRNA averaged 55.8 ± 6.4 fg ($n = 5$) and was increased by 63.6% to 91.3 ± 12.3 fg ($n = 4$) ($P < 0.05$) at 3 weeks after the induction of diabetes with streptozotocin (Figure 4).

Prevention of Mesenteric Hypertrophy by the NHE Inhibitor Cariporide

We assessed biochemical and physiological parameters as well as total mesenteric weight at 3 weeks, and these data are summarized in Table 2. Body weight in diabetic animals was reduced, but cariporide had no significant effect on body weight. Diabetes was associated with an increase in blood glucose that was not influenced by cariporide treatment. There was no significant difference in the systolic blood pressure between control and diabetic animals, and this also was not influenced by cariporide treatment. Cariporide significantly ($P < 0.01$) attenuated the increase in mesenteric weight in diabetic animals (Figure 5A). Mesenteric media/lumen ratio was increased in diabetes and was significantly reduced ($P < 0.0001$) by treatment with cariporide to levels approaching those in control rats (Figure 5B). Cariporide treatment did not affect these parameters in control rats.

ECM accumulation, as assessed by trichrome staining, was increased in both the medial and adventitial layers of mesenteric vessels of diabetic rats (Figure 5C). Cariporide treatment was associated with less ECM accumulation than in the untreated diabetic rats in both vessel layers, and cariporide treatment did not alter the amount of ECM in control rats (Figure 5C). Representative sections of trichrome-stained mesenteric arterioles from each of the 4 experimental groups are shown in Figure 6.

Discussion

The streptozotocin-induced diabetic rat is a model of mesenteric vascular hypertrophy without physical damage to the vessels; at a cellular level, the hypertrophy is due to both increased smooth muscle cell mass and ECM. We have used this model to demonstrate that the development of the vascular hypertrophy is preceded by activation of NHE in the vascular smooth muscle and that the administration of an inhibitor of NHE activity, cariporide, prevents the development of the hypertrophy. It is important to appreciate that the vascular changes observed in this model do not represent atherosclerosis but may be important as early manifestations of diabetes-associated vascular disease.

In this study, as a prelude to the investigation of NHE activity in the hypertrophying vessels, we confirmed the time course of the development of mesenteric hypertrophy after streptozotocin administration to adult rats. We observed no increase in mesenteric weight by 1 week but a 56% increase at 3 weeks after treatment.

Previous studies investigating NHE activity in diabetes have used either blood-borne cells⁹ or cultured VSMCs exposed to high glucose.¹⁶ We used isolated, intact blood vessels mounted under tension to study NHE activity after the

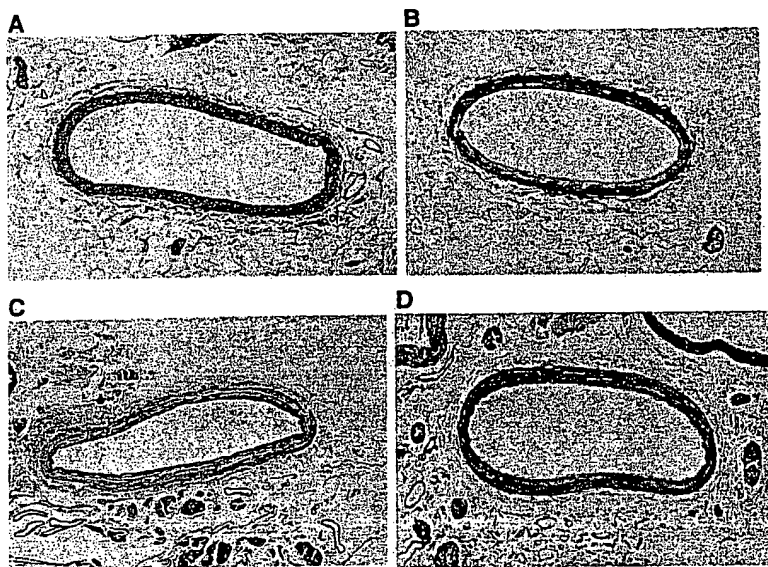


Figure 6. Examination of ECM deposition in diabetic vessels. Adult rats with or without streptozotocin-induced diabetes for 3 weeks were treated with vehicle or the NHE inhibitor cariporide (100 mg/kg, 3 times daily by gavage), and vessels were subjected to trichrome staining as detailed in Materials and Methods. Representative mesenteric arterioles from rats in control (A), control plus cariporide (B), diabetic (C), and diabetic plus cariporide (D) groups are shown.

vessels have been exposed to the full diabetic milieu. Our data show that manipulation of the metabolic environment in intact animals can alter NHE activity in blood vessels. The mean resting pH_i of mesenteric arteries from diabetic rats was significantly higher than for control rats, as was the rate of recovery from acid loading (V_{\max}) (see Table 1). This increase in apparent V_{\max} could not be explained by an alteration in intrinsic cellular buffering, because this parameter was the same in control and diabetic vessels, indicating that the increased V_{\max} reflects increased activity or increased abundance of the glycoprotein transporter. Furthermore, we demonstrated that the expression of NHE-1 mRNA was increased by >60% at 3 weeks after treatment with streptozotocin.

The maximum acidification achievable in the mesenteric arteries (see Table 1) was considerably less than that in studies of cultured cells^{6,19} but was consistent with previous reports from studies in blood vessels.^{23–25} The level of acidification was sufficient to cause maximum activation of the pH recovery process (see Figure 3, which shows a distinctive sigmoidal shape with a plateau at maximal recovery). Hence, and importantly, our methodology ensured that the measurement represents maximal NHE activity for all groups of vessels studied.

It has previously been shown that hypertrophy observed in vessels from diabetic rats arises from an increase in the medial cross-sectional area.²⁶ The possible mechanisms for this increase of the cross-sectional area (vascular hypertrophy) are VSMC hypertrophy, cellular hyperplasia, and increased ECM. Previously it has been shown that there is no change in the size of the smooth muscle cells when digested from the diabetic mesenteric vessels.²⁶ Previous studies have suggested not only an increase in ECM in diabetic vessels²¹ but also a change in the type of fibrillar collagens that are accumulating in the medial layer of the mesenteric vessels from diabetic rats. The present study confirms this increase in ECM accumulation in the diabetic vessels and notes that this is observed in both the medial and adventitial layers.

Our data are consistent with a number of *in vitro* studies, which also show an association between growth and increased NHE activity in diabetes. For example, diabetes is characterized by renal hypertrophy, and concomitant with this growth response is an elevation of NHE activity in the luminal membrane of the proximal tubule.²⁷ Significantly, NHE activity was elevated in the mesenteric arteries of diabetic rats within 1 week of onset of diabetes, before vascular hypertrophy is apparent.¹⁷ This implies that this change in NHE activity is not a manifestation of the trophic response in diabetes, but it may be an important pathogenic mechanism in this response.

To discern the underlying basis for the increased transport activity found in the mesenteric arteries of diabetic rats, we examined the kinetic characteristics of NHE. Altered NHE activity in the mesenteric arteries may result from a change in either the apparent V_{\max} , K_m , or in the Hill coefficient, the latter reflecting an alteration in the properties of the proton modifier site. In the present experiments, kinetic analyses of NHE in mesenteric arteries revealed an enhanced maximal velocity (V_{\max}) of this exchanger with no change either in the apparent affinity (K_m) for H⁺ or in the activation of the H⁺ modifier site (Hill coefficient). This pattern of NHE activation suggests that diabetes induces increased expression of NHE and is consistent with *in vitro* studies of diabetic tissues, which found an increase in NHE V_{\max} but no change in K_m .^{5,28} Accordingly, we were able to confirm activation of NHE-1 synthesis by demonstrating a substantial (>60%) increase in the expression of NHE-1 mRNA in mesenteric vessels of diabetic rats.

The entire circulation in our animals was, of course, exposed to the hyperglycemic stimulus for prolonged periods. The fact that activation of NHE activity was found in mesenteric arteries of diabetic rats may suggest that hyperglycemia alone is sufficient to activate the antiporter *in vivo*, but this may not mean that the response is direct. Vascular-derived growth factors both activate NHE⁸ and increase the

steady-state levels of NHE-1 mRNA.²⁹ Transforming growth factor- β_1 gene expression is elevated in the mesenteric arteries of diabetic rats within 1 week,³⁰ which also corresponds to the rise in NHE activity found in this study and to the rise in specific activity of PKC and diacylglycerol levels in diabetic tissues.³⁰ Hyperglycemia also activates expression of vascular endothelial growth factor in VSMCs,³¹ so it is possible that other growth factors may be responsive to the glycemic environment. Rises in both growth factor expression and NHE activity occur before any significant vascular growth and may represent an early response to diabetes, which then leads to vascular remodeling. Our results showing significant elevation in NHE-1 gene expression in the diabetic model are consistent with the results of Rao et al,²⁹ who demonstrated induction by growth factors, and Williams and Howard,¹⁶ who demonstrated induction due to high glucose in cultured VSMCs.

As a cell membrane system, the NHE is subject to pharmacological inhibition. Inhibitors interact with the substrate (sodium) binding site on the exchanger to inhibit activity independent of any operating mechanisms of activation. Amiloride derivatives have been shown to inhibit VSMC proliferation under physiological conditions in vitro, and 2 distinct classes of the NHE inhibitors have been shown to inhibit the development of vascular wall thickening in the rat carotid artery injury model. We chose cariporide to investigate the potential role of NHE inhibition in preventing the development of the mesenteric hypertrophy. Hypertrophy was assessed grossly, as the total weight of the mesentery, and the contribution of medial thickening was also quantified. The data confirmed our earlier finding of increased weight and media after streptozotocin administration and clearly showed that inhibition of NHE with cariporide inhibited the development of the vascular hypertrophy. Furthermore, the data indicate that cariporide did not mediate its antitrophic action via effects on metabolic pathways such as glucose or via hemodynamic pathways such as blood pressure, 2 major pathways in the pathogenesis of diabetic vascular disease.^{17,32}

Hyperactivity of the Na⁺-H⁺ antiporter has been implicated in the pathogenesis of vascular disease in both hypertension and diabetes.⁵⁻⁷ Studies in vitro show that high glucose induces both increased VSMC proliferation^{13,15} and, in the presence of serum, activation of NHE-1.¹⁶ The present in vivo study shows that an early and sustained NHE activation is observed in the mesenteric vasculature. We have further shown that administration of the NHE inhibitor during the onset and early phase of the development of hyperglycemia prevents the development of the vascular hypertrophy without influencing glycemic control. These results clearly show that inhibition of NHE is potentially a target for therapeutic intervention in the rapid development of vascular disease in diabetes.

The reduction in vascular hypertrophy observed with cariporide may be due to a reduction in proliferation, cell hypertrophy, or a reduction in ECM accumulation. Although not all mechanisms were extensively explored in this study, as previously reported a major explanation for the increase in vascular weight and wall/lumen ratio in diabetes relates to ECM accumulation. The link between

NHE and ECM accumulation has not been previously investigated in detail. Indeed, NHE activity has been closely linked to cell proliferation, a phenomenon that, although present in this model, appears to be transient and primarily occurs in the endothelial and adventitial layers rather than the smooth muscle cell layer. The lack of the effect of cariporide in control rats suggests that blockade of NHE mediates an effect specific for diabetes. It remains to be determined how NHE activation is linked to matrix deposition, and this warrants further exploration. This would allow us to investigate how NHE-1 inhibition may lead to reduced matrix deposition in diabetes.

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